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WO 02/052021 A1

(54) **Title:** THERMOSTABLE L-ARABINOSE ISOMERASE AND PROCESS FOR PREPARING D-TAGATOSE THEREBY

(57) **Abstract:** The present invention relates to a novel gene coding for L-arabinose isomerase derived from Thermotoga neapolitana 5068, a thermostable arabinose isomerase expressed from the said gene, a recombinant expression vector containing the said gene, a microorganism transformed with the said expression vector, a process for preparing thermostable arabinose isomerase from the said transformant and a process for preparing D-tagatose employing the said enzyme. Since the recombinant arabinose isomerase_of the invention is highly thermostable and can produce tagatose with high yield at high temperature, it can be efficiently applied in pharmaceutical and food industries.

THERMOSTABLE L-ARABINOSE ISOMERASE AND PROCESS FOR PREPARING D-TAGATOSE THEREBY

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a thermostable arabinose isomerase and a process for preparing tagatose using the same, more specifically, to a noble gene coding for L-arabinose isomease derived from *Thermotoga neapolitana* 5068, a thermostable arabinose isomerase expressed from the said gene, a recombinant expression vector containing the said gene, a microorganism transformed with the said expression vector, a process for preparing thermostable arabinose isomerase from the said transformant and a process for preparing D-tagatose employing the said enzyme.

Background of the Invention

In recent years, growing concerns about health have led much research effort to the development of healthful foods. As one of the above efforts, sugar alcohols have been proposed as sweeteners which can substitute sugar, known to cause adult diseases, and are practically being used. Since the said sweeteners are known to have adverse side effects such as causing diarrhea when ingested more than certain amount, there is an urgent need to develop substitutional sweeteners without harmful effects.

Among substitutional sweeteners which have little side effect, tagatose, a keto-sugar of galactose, has similar sweetness to D-fructose, and has known not to be absorbed or metabolized in the body, making tagatose a safe low-caloric substitutional sweetener for sugar. Also, it has been reported that tagatose can be employed as an

intermediate for the preparation of useful optically active isomers, detergents and cosmetics, also, as an additive or raw material for the synthesis of drugs, especially, its ability to lower blood sugar level renders tagatose a therapeutic and preventive agent for diabetes, and a low caloric diet agent.

Currently, tagatose is mostly prepared via chemical synthesis from galactose(see: USP 5,002,612), which comprises the steps of isomerization of galactose catalyzed by metal hydroxide in the presence of inorganic salts to form an intermediate of metal hydroxide-tagatose complex, and neutralization of the complex by adding acid to yield final product, tagatose.

Alternative method for manufacturing tagatose is an enzymatic method in which galactose is converted into tagatose via conversion of aldose or aldose derivatives into ketose or ketose derivatives. Especially, it has been reported that arabinose isomerase which catalyzes the conversion reaction of L-arabinose into L-ribulose can be employed for production of tagatose in vitro using galactose as a substrate. However, the yield of tagatose produced by arabinose isomerase from galactose is as low as 20%, hindering industrial application of conversion process of galactose into tagarose. Although the method for manufacturing tagatose from milk or cheese has been developed(see: USP 6,057,135), again, low yield is the limitation for its industrial use.

Under the circumstances, there are strong reasons for exploring and developing a novel enzyme which can produce tagatose with high yield.

Summary of the Invention

The present inventors have made an effort to develop an enzyme which can produce tagatose with high yield, thus, have found that tagatose can be produced with high yield from galactose by employing a recombinant arabinose

isomerase produced from *E. coli* transformed with recombinant vector containing arabinose isomerase gene derived from *Thermotoga neapolitana* 5068.

The first object of the present invention is, therefore, to provide arabinose isomerase gene derived from *Thermotoga neapolitana* 5068.

The second object of the invention is to provide arabinose isomerase expressed from the gene.

The third object of the invention is to provide a recombinant expression vector containing the arabinose isomerase gene.

The fourth object of the invention is to provide a recombinant *E. coli* transformed with the recombinant expression vector.

The fifth object of the invention is to provide a process for preparing recombinant arabinose isomerase using the transformed *E. coli*.

The sixth object of the invention is to provide a process for preparing tagatose from galactose using the enzyme.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

Figure 1 is a schematic diagram showing the construction strategy of an expression vector containing arabinose isomerase gene of the invention.

Figure 2 is a graph showing activity profile of arabinose isomerase of the invention depending on temperature.

Figure 3 is a graph showing thermostability of arabinose isomerase of the invention.

Figure 4 is a graph showing the time course of conversion rate of galactose into tagatose by arabinose isomerase of the invention at various reaction temperatures.

Figure 5 is a graph showing the time course of changes in thermostability of immobilized arabinose isomerase of the invention.

DETAILED DESCRIPTION OF THE INVENTION

To prepare thermophilic or thermostable arabinose isomerase for industrial use, the present inventors have cloned a gene coding for arabinose isomerase from genomic DNA of *Thermotoga neapolitana* 5068(DSM 5608) and analyzed nucleotide sequence and deduced amino acid sequence from the said gene. The nucleotide sequence and deduced amino acid sequence of the gene encoding arabinose isomerase of the invention(SEQ ID NO: 3) has shown to have 83.2% and 94.8% homology, respectively, to those of the putative arabinose isomerase gene of *Thermotoga maritima* of which entire nucleotide sequence has been verified via genome project.

For high level expression of the said cloned arabinose isomerase in *E. coli*, the gene coding for the enzyme was inserted into an expression vector pET22b(+)(Novagen, U.S.A.) to construct a recombinant expression vector pTNAI, which was then introduced into *E. coli* BL21. The transformed recombinant *E. coli* was named "*E. coli* BL21/DE3(pTNAI)" and deposited with an international depository authority, the Korean Culture Center of Microorganisms(KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea) on December 4, 2000 as accession no. KCCM-10231.

The said *E. coli* BL21/DE3(pTNAI) was grown to obtain recombinant arabinose isomerase, which was characterized to have optimum pH of 7.0, optimum reaction temperature of 85°C. Furthermore, over 80% of remaining activity was

measured after 2 hour heat treatment at 80°C, indicating that the enzyme is exceedingly heat stable.

Tagatose can be produced by employing arabinose isomerase of the invention prepared from *E. coli* transformed with a recombinant expression vector containing the gene for arabinose isomerase derived from *Thermotoga* sp., and galactose as a substrate, under a condition of pH 5 to 8, more preferably pH 6 to 8, most preferably pH 7, and 60 to 100°C, more preferably 70 to 95°C, most preferably 85°C.

Aqueous solution of galactose was subjected to isomerization reaction employing recombinant arabinose isomerase of the invention, and it has been found that conversion rate into tagatose was over 68% at 80°C.

When the said recombinant arabinose isomerase is employed for industrial production of tagatose, soluble form of the enzyme may be employed, nevertheless, it is more preferable to immobilize the enzyme on the beads used in industry. For example, in case of the recombinant arabinose isomerase of the invention immobilized on silica beads, the remaining activity was measured to be over 80% of original activity after 20 day-heat treatment at 90°C, thus, it can be applied for thermal process over 80°C in industry.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Cloning of arabinose isomerase gene

Thermotoga neapolitana 5068(DSM 5068) was grown under an anaerobic condition and cells were harvested by centrifugation at 8000xg for 10 minutes. Genomic DNA isolated from the cells harvested above was partial digested with Sau3AI(TaKaRa Biotechnology, Japan) to obtain 12kb or shorter fragments of DNA. The DNA fragments

were inserted into ZAP Expression Vector(Stratagene, U.S.A.) and packaged to prepare a genomic library of *Thermotoga neapolitana* 5068. Nucleotide sequences of the genes for conventional thermophilic or thermostable arabinose isomerase were analyzed to prepare primer araAF: 5'-ATGATCGATCTCAAACAGTATGAG-3'(SEQ ID NO: 1) and primer araAR: 5'-TCATCTTTTTAAAAGTCCCC-3'(SEQ ID NO: 2), which were used in PCR for the preparation of probes for DNA-DNA hybridization. The genomic library prepared above was screened for DNA fragments containing arabinose isomerase gene by DNA-DNA hybridization to obtain a recombinant vector containing a gene encoding arabinose isomerase of *Thermotoga neapolitana* 5068. The nucleotide sequence of arabinose isomerase gene(SEQ ID No: 3) cloned above and the deduced amino acid sequence(SEQ ID No: 4) from the said gene were compared with those of known arabinose isomerase genes, respectively(see: Table 1).

Table 1: Comparison of homology between arabinose isomerase of the invention and known arabinose isomerases

Strain	Gene Sequence (homology, %)	Amino Acid Sequence (homology, %)
Thermotoga maritima	83.2	94.8
Bacillus stearothermophilus	61.9	62.8
Bacillus halodurans	59.1	59.0
Bacillus subtilis	58.6	55.5
Salmonella typhimurium	57.8	54.5
Escherichia coli	59.0	54.3
Mycobacterium smegmatis	56.3	50.7

As shown in Table 1, it has been found that the arabinose isomerase of the invention is a novel enzyme which has 83.2% homology of nucleotide sequence and 94.8%

homology of amino acid sequence to the sequences of published putative arabinose isomerase of *Thermotoga maritima*, respectvely.

Example 2: Preparation of recombinant expression vector and recombinant *E. coli*

In order to obtain high level expression of the said thermostable arabinose isomerase in *E. coli* using the arabinose isomerase gene obtained in Example 1, the said gene was inserted into an expression vector pET 22b(+)(Novagen, U.S.A.) double-digested with *NdeI* and *EcoRI* to construct a recombinant expression vector pTNAI(see: Figure 1), which was then introduced into *E. coli* BL21. The transformed recombinant *E. coli* was named "*E. coli* BL21/DE3(pTNAI)" and deposited with an international depository authority, the Korean Culture Center of Microorganisms(KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea) on December 4, 2000 as accession no. KCCM-10231.

Example 3: Expression of recombinant arabinose isomerase

The recombinant *E. coli* BL21/DE3(pTNAI)(KCCM-10231) prepared in Example 2 was inoculated into LB(Luria-Bertani) medium(1% v/v) and incubated at 37°C for 2 hours, to which lactose was added to a final concentration of 1mM and expression of recombinant arabinose isomerase was induced for 12 hours. For assay of expressed arabinose isomerase, cells were collected by centrifugation at 8000xg for 10 minutes, resuspended in 10ml of 100mM MOPS buffer(4-morpholinepropanesulfonic acid, pH 7.0), and then disrupted by sonication to obtain crude enzyme, with which galactose isomerization reaction was carried out. Galactose isomerization was performed by mixing 100μl of the said crude enzyme solution with 40mM(final concentration) galactose as a substrate, followed by

adding 1ml of enzyme reaction buffer(100mM MOPS buffer, pH 7.0) containing cofactors(1mM $MnCl_2$, 1mM $CoCl_2$) and incubating at 85°C for 20 minutes. The product of the above reaction was detected using cysteine-carbazole-sulfuric acid method(<u>see</u>: Dische, Z., and E. Borenfreund., A New Spectrophotometric Method for the Detection and Determination of Keto Sugars and Trioses, *J. Biol. Chem.*, 192:583-587, 1951), and it has been found that normal galactose isomerization has been undergone.

<u>Example 4</u>: Purification of recombinant arabinose isomerase

For purification of recombinant arabinose isomerase expressed by the method described in Example 3, cells were collected by centrifugation at 8000xg for 19 minutes and cell wall of *E. coli* was disrupted by sonication, which was followed by centrifugation at 20,000xg for 20 minutes to obtain supernatant. Then, the said supernatant was heat-treated at 85°C for 20 minutes, centrifuged at 20,000xg for 20 minutes to get rid of precipitate, and the supernatant was further purified by ammonium sulfate-mediated precipitation and finally ion-exchange column chromatography(Q-Sepharose Fast Flow, Pharmacia, Sweden). pH dependancy of the said purified enzyme was analyzed and optimum pH was found to be around 7.0.

<u>Example 5</u>: Optimum pH and optimum temperature of recombinant arabinose isomerase

Activity of the purified recombinant arabinose isomerase prepared in Example 4 was analyzed on galactose substrate and optimum pH was found to be around 7.0. Optimum temperature for isomerization reaction was determined using the same method as described in Example 3. The tested reaction temperatures for galactose isomerization were 60, 70, 75, 80, 85, 90 and 100°C, and maximum activity was obtained around 85°C(<u>see</u>: Figure 2).

Example 6: Thermostability of recombinant arabinose isomerase

To assess the thermostability of recombinant arabinose isomerase of the invention, crude enzyme prepared in Example 3 was heat-treated at 60, 70, 80 and 90°C for 10, 20, 30, 60, 90 and 120 minutes respectively, and remaining activity of recombinant arabinose isomerase for isomerization was determined as described in Example 3(see: Figure 3). As shown in Figure 3, it has been found that over 80% of enzyme activity was remained after 2 hour heat-treatment at 80°C.

Example 7: Conversion rate of galactose into tagatose at various temperature

By employing recombinant arabinose isomerase of the invention, the conversion rate of galactose into tagatose was determined at various temperatures and various time points. Substrate used was 10mM galactose instead of 40mM galactose in enzyme reaction mixture in Example 3. After incubation at 60, 70, 80 and 90°C for 20 hours, tagatose yield was determined employing BioLC(see: Table 2 and Figure 4).

Table 2: Conversion rate of galactose into tagatose at various temperature

Enzyme Reaction Temperature	60°C	70°C	80°C	90°C
Conversion Rate into Tagatose	31.7	40.4	68.1	57.4

As shown in Table 2 and Figure 4, the higher the reaction temperature was, the higher tagatose yield was obtained, and conversion rate into tagatose was as high as 68% at 80°C.

Example 8: Immobilization of arabinose isomerase and improvement of thermostability

Arabinose isomerase was immobilized on silica beads, heat-treated under an aqueous condition at 90°C and the remaining activity was determined at various time points(see: Figure 5). As shown in Figure 5, remaining activity of the immobilized enzyme was over 80% after 20 day-heat treatment at 90°C and over 60% after 30 day-heat treatment, indicating that the immobilized arabinose isomerase of the invention can be applied for thermal process in industry.

As clearly illustrated and demonstrated above, the present invention provides a noble gene coding for L-arabinose isomease derived from *Thermotoga neapolitana* 5068, a thermostable arabinose isomerase expressed from the said gene, a recombinant expression vector containing the said gene, a microorganism transformed with the said expression vector, a process for preparing thermostable arabinose isomerase from the said transformant and a process for preparing D-tagatose employing the said enzyme. Since the recombinant arabinose isomerase of the invention is highly thermostable and can produce tagatose with high yield at high temperature, it can be efficiently applied in pharmaceutical and food industries.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in description

On page 4 , lines 23-33 and page 7 , lines 8-20 .

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on additional sheet

Name of depositary institution

Korean Culture Center of Microorganisms(KCCM)

Address of depositary institution (including postal code and country)

Korean Culture Center of Microorganisms(KCCM)
361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu
Seoul, 120-091, Republic of Korea

Date of deposit	Accession Number
Dec. 04, 2000	KCCM-10231

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information continues on an additional sheet

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
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Authorized officer	Authorized officer

What is Claimed is:

1. A gene coding for arabinose isomerase having a nucleotide sequence of SEQ ID NO: 3 which is derived from *Thermotoga* sp.

2. A gene coding for arabinose isomerase having a nucleotide sequence of SEQ ID NO: 4 which is derived from the nucleotide sequence of claim 1.

3. A recombinant expression vector pTNAI represented as a genetic map of Fig. 1 which contains the nucleotide sequence of SEQ ID NO: 3 of a gene derived from *Thermotoga* sp.

4. *E. coli* BL21/DE3(pTNAI)(KCCM-10231) transformed with the recombinant expression vector pTNAI of claim 3.

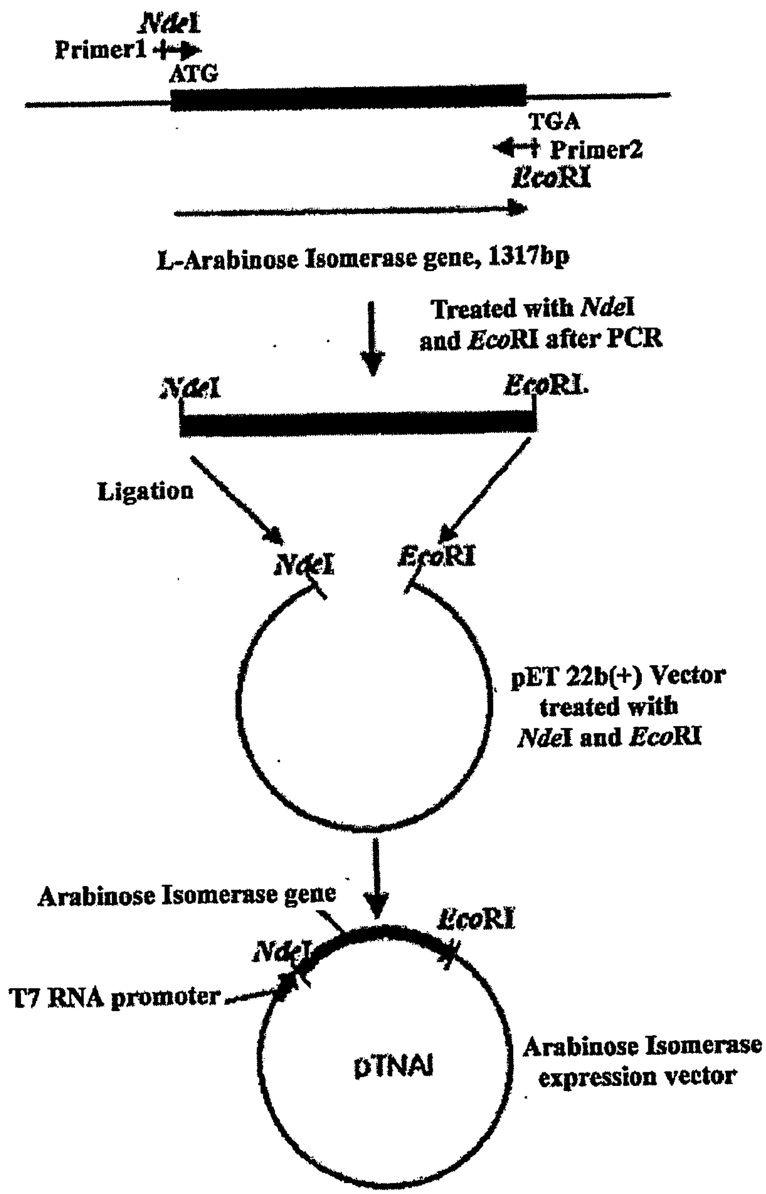
5. A process for preparing a recombinant arabinose isomerase which comprises a step of culturing a microorganism transformed with a recombinant expression vector containing the gene for arabinose isomerase derived from *Thermotoga* sp. of claim 1 to obtain a recombinant arabinose isomerase from the culture.

6. The process for preparing a recombinant arabinose isomerase of claim 5, wherein the microorganism transformed with a recombinant expression vector containing the gene for arabinose isomerase is *E. coli* BL21/DE3(pTNAI)(KCCM-10231).

7. A process for preparing tagatose which comprises a step of reacting arabinose isomerase prepared from a microorganism transformed with a recombinant expression vector containing a gene for arabinose isomerase derived from *Thermotoga* sp. with a substrate of galactose under a condition of pH 5 to 8 and 50 to 100°C to obtain tagatose.

8. The process for preparing tagatose of claim 7, wherein the microorganism transformed with a recombinant expression vector containing a gene for arabinose isomerase derived from *Thermotoga* sp. is *E. coli* BL21/DE3(pTNAI) (KCCM-10231).

9. The process for preparing tagatose of claim 7, wherein the arabinose isomerase is immobilized recombinant arabinose isomerase.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Ligation
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EcoRI
pET 22b(+) Vector
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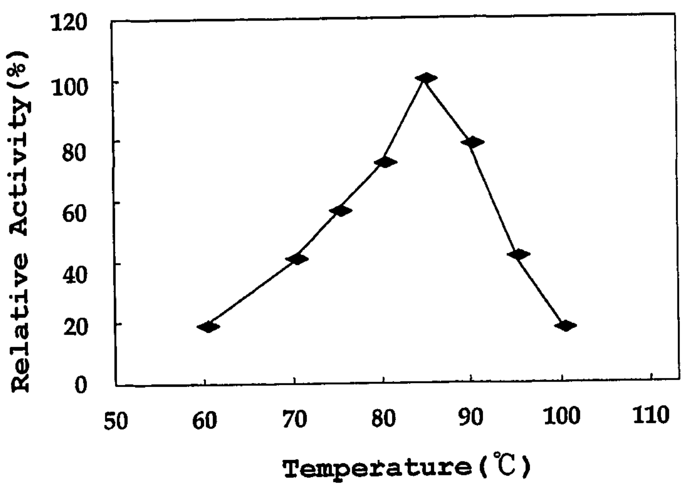
Fig. 1



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Temperature(℃)

Fig. 2

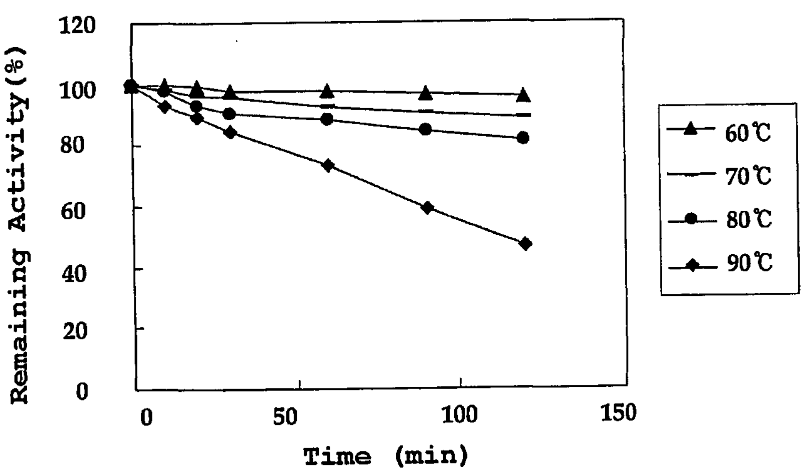

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Fig. 3

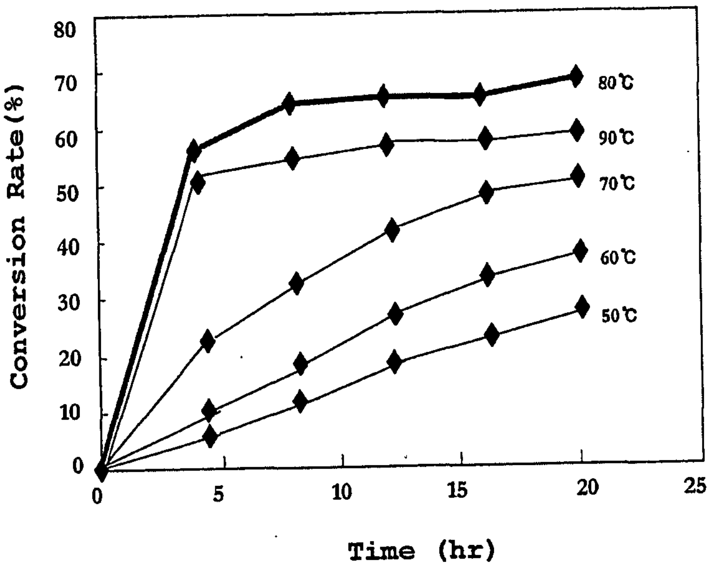
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Fig. 4

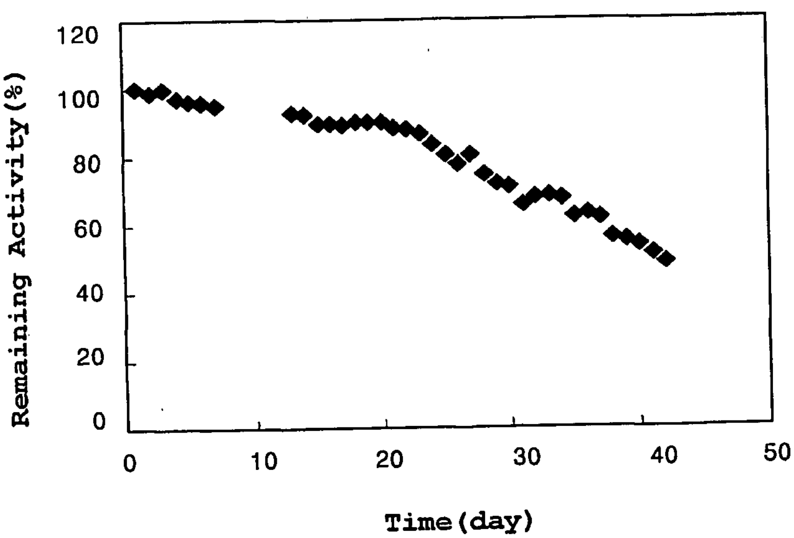
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Fig. 5

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Gly Asp Arg Glu His Gly Phe Ile His Ala Arg Met Arg Leu Pro Arg
130 135 140

Lys Val Val Val Gly His Trp Glu Asp Arg Glu Val Arg Glu Lys Ile
145 150 155 160

Ala Lys Trp Met Arg Val Ala Cys Ala Ile Gln Asp Gly Arg Thr Gly
165 170 175

Gln Ile Val Arg Phe Gly Asp Asn Met Arg Glu Val Ala Ser Thr Glu
180 185 190

Asp Asp Lys Val Glu Ala Gln Ile Lys Leu Gly Trp Ser Ile Asn Thr
195 200 205

Trp Gly Val Gly Glu Leu Ala Glu Gly Val Lys Ala Val Pro Glu Asn
210 215 220

Glu Val Glu Glu Leu Leu Lys Glu Tyr Lys Glu Arg Tyr Ile Met Pro

225 230 235 240

Glu Asp Glu Tyr Ser Leu Lys Ala Ile Arg Glu Gln Ala Lys Met Glu
245 250 255

Ile Ala Leu Arg Glu Phe Leu Lys Glu Lys Asn Ala Ile Ala Phe Thr
260 265 270

Thr Thr Phe Glu Asp Leu His Asp Leu Pro Gln Leu Pro Gly Leu Ala
275 280 285

Val Gln Arg Leu Met Glu Glu Gly Tyr Gly Phe Gly Ala Glu Gly Asp
290 295 300

Trp Lys Ala Ala Gly Leu Val Arg Ala Leu Lys Val Met Gly Ala Gly
305 310 315 320

Leu Pro Gly Gly Thr Ser Phe Met Glu Asp Tyr Thr Tyr His Leu Thr
325 330 335

Pro Gly Asn Glu Leu Val Leu Gly Ala His Met Leu Glu Val Cys Pro
340 345 350

Thr Ile Ala Lys Glu Lys Pro Arg Ile Glu Val His Pro Leu Ser Ile
355 360 365

Gly Gly Lys Ala Asp Pro Ala Arg Leu Val Phe Asp Gly Gln Glu Gly
370 375 380

Pro Ala Val Asn Ala Ser Ile Val Asp Met Gly Asn Arg Phe Arg Leu
385 390 395 400

Val Val Asn Arg Val Leu Ser Val Pro Ile Glu Arg Lys Met Pro Lys
405 410 415

Leu Pro Thr Ala Arg Val Leu Trp Lys Pro Leu Pro Asp Phe Lys Arg
420 425 430

Ala Thr Thr Ala Trp Ile Leu Ala Gly Gly Ser His His Thr Ala Phe
435 440 445

Ser Thr Ala Val Asp Val Glu Tyr Leu Ile Asp Trp Ala Glu Ala Leu
450 455 460

Glu Ile Glu Tyr Leu Val Ile Asp Glu Asn Leu Asp Leu Glu Asn Phe
465 470 475 480

Lys Lys Glu Leu Arg Trp Asn Glu Leu Tyr Trp Gly Leu Leu Lys Arg
485 490 495

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/02243

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/61, C12N 9/90, C12N 15/63, C12N 1/20, C12P 19/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/61, C12N 9/90, C12N 15/63, C12N 1/20, C12P 19/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the interntational search (name of data base and, where practicable, search terms used)

NCBI, PubMed, CA, USPTO, PAJ, Espacenet, "Thermotoga", "arabinose isomerase", "tagatose"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A Y	Nelson, K.E. et al., "Evidence for lateral gene transfer between Archaea an bacteria from genome sequence of Thermotoga mariti", Nature, 399(6734), 323-29, 1999 & NCBI Accession # AE001709.	1-6, 8, 9 7
Y	Roh, H.J. et al., "Bioconversion of D-galactose into D-tagatose by expression of L-arabinose isomerase", Biotechnol. Appl. Biochem., 31(1), 1-4, 2000.	7
Y	US 6,057,135 A (Kraft-Foods, Inc.), 02 May 2000.	7
Y	WO 2000/068397 A1 (Tongyang Confectionery Co.), 16 Nov. 2000.	7

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand *the principle or theory underlying the invention*
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 MARCH 2002 (13.03.2002)	13 MARCH 2002 (13.03.2002)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR01/02243

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6,057,135 A	02 May 2000	EP 552,894 A2	28 Jul. 1993